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MOLECULAR COMPUTATION WITH AUTOMATED MICROFLUIDIC SENSORS (MCAMS)

Princeton University

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We combined microfluidic technology with recently developed algorithms of nucleic acid-based computing to demonstrate a compact, automated nucleotide-based computational device capable of rapid detection of computational output. We explored both experimental and theoretical aspects of the construction of microfluidic nucleic acid computing. Microreactors implementing Boolean operators lent themselves to a relatively simple implementation of DNA computing. On the theoretical side, we simulated the performance of individual reactors and designed small reactor systems ("sorters") mimicking AND and OR gates and systems capable of solving complex Boolean expressions. We compared positive vs. negative selection and parallel vs. series arrangements of reactors, considering both the accuracy of and disproportionate representation ("bias") within the final pool of solutions. We also developed $O(P2)$ software that designs operating instructions for an $O(NL)$ system capable of solving any Boolean expression.

We developed software for automated data collection and signal analysis of electronic detection of labeled DNA and explored novel nanocrystal probes that enable detection of DNA for readout in on-chip electrical assays. We focused in the areas of methods to produce nanocrystals bearing precise numbers of oligonucleotides, and development of nanocrystal precise groupings that bear a collective signature.

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SUMMARY

The main objective of this research is to combine microfluidic technology with recently developed algorithms of nucleic acid-based computing to create a compact, automated nucleotide-based computational device capable of rapid detection of the computational output.

Princeton microreactors implementing Boolean operators lend themselves to a relatively simple implementation of DNA computing. Like electronic logic gates, they can be arranged like building blocks to implement complex expressions, which can be solved using either negative or positive selection. We have explored both experimental and theoretical aspects of the construction of microfluidic nucleic acid computing.

On the theoretical side, Princeton simulated the performance of individual reactors and designed small reactor systems ("sorters") mimicking AND and OR gates and systems capable of solving complex Boolean expressions. We compared positive vs. negative selection and parallel vs. series arrangements of reactors, considering both the accuracy of and disproportionate representation ("bias") within the final pool of solutions. We also developed $O(P^2)$ software that designs operating instructions for an $O(NL)$ system capable of solving any Boolean expression.

Stanford developed software for automated data collection and signal analysis of electronic detection of labelled DNA.

Berkeley developed novel nanocrystal probes that enable detection of DNA for readout in on-chip electrical assays. Efforts have focused in the areas of methods to produce nanocrystals bearing precise numbers of oligonucleotides, and development of nanocrystal precise groupings that bear a collective signature.

Princeton University, Landweber Laboratory:

Introduction: Microflow technology is an important tool to realize molecular computing (Gehani and Reif, 1999; van Noort *et al.*, 2002a) with DNA, RNA or proteins. The advantages with this technology include the use of small (nanoliter) volumes of solutions and increased reaction rates due to reduced diffusion time. Microflow structures provide control over the flow of information. Such structures can be designed to be problem-specific or re-configurable (McCaskill, 2000; van Noort *et al.*, 2001). Computational problems can in principle be solved on a network of these reactors executing a series of logic operations.

In addition to developing new technologies for DNA computing, it is also useful to translate them into all-purpose mathematical tools. Since every mathematical problem can be written as a set of Boolean expressions, Livstone designed components that could implement three functions sufficient for composing every Boolean expression: AND and OR (by proper arrangement and operation of microreactors) and NOT (by algebraic transformation). These components consist of multiple reactors (with heaters) arranged in series. With proper coordinated operation of the heaters, linear arrays of reactors can solve arbitrarily complex Boolean expressions in polynomial time.

Methods: Several methods of DNA computing have been described previously (e.g. Faulhammer *et al.*, 2000; Winfree, 2000; Suyama, 2002)). Our group has presently opted for single-stranded DNA (ssDNA) "capture probes" (CPs) immobilized in microreactors which bind to and select specific sequences from a combinatorial pool of all possible solutions. This pool flows through the reactor system, thereby implementing a logical operation. The systems are produced in Polydimethylsiloxane (PDMS, Sylgard 184, Dow-Corning, MI) and recently in glass by HF etching techniques.

We have explored two methods of selection. In negative selection, incorrect solutions are removed by binding within microreactors. While the microfluidic structure is relatively simple and straightforward, the error rate will be higher as not all unwanted solution strands will be captured (see below). In positive selection, correct solutions are recovered by binding within microreactors; theoretically, this allows near-perfect accuracy, but may involve large losses of material. Also, captured strands must be released from the CPs, which makes the system more complex. Micro thermoelectric modules were tested to regulate the temperature, and therefore binding, in microfluidic system.

Capture of ssDNA within a reactor was modelled as a bimolecular reaction occurring in consecutive slices of the reactor. To evaluate the efficacy of AND and OR sorters, we first prioritized high <u>accuracy</u> of the method–the percentage of recovered molecules which represent correct solutions to the expression–and then low "<u>bias</u>," or disproportionate recovery of "correct"

molecules. The assumptions were: (i) Molecules bind only their complements, not non-complementary sequences or non-specifically to surfaces, and (ii) Binding efficiency is constant in all microreactors regardless of sequence. (Livstone and Landweber, 2003)

Results: Biotinylated CP's have been immobilized to streptavidin-functionalized polystyrene beads. Initially, the beads were immobilized to the surface of the reactor itself (van Noort *et al.*, 2002b), but now bead barriers are used to prevent the beads from flowing out of the reactor. At first, the barriers were fixed, but later they were replaced with PDMS valves as in Ungar *et al.*, 2000 (see Fig. 1.1a). With valves, beads can be loaded through the same channel as the applied solution, while the valves are used to control the distribution of beads, so no dedicated bead delivery channels are needed. Furthermore, the valve system is a completely different circuit from the flow structure, which means there will be no interference with the flow due to the structure, as is the case with bead delivery channels (see Fig. 1.1b). After the computation has been performed, the beads can be flushed out by opening all the valves, making the structure reusable and therefore reprogrammable.

Negative selection modules
To prove that negative selection is feasible, a long meander (a 50 µm wide channel filled with 5.6 µm beads) was fabricated to monitor the effect of hybridization reduction over a distance (over a length of 20 mm). Initially 10 µl of 0.5 µM d(A)25 solution was injected. By comparing the maximal intensities of hybridization, as measured for different concentrations, we observed that towards 2/3 of the channel the concentration dropped from µM to fM scale, 9 orders of magnitude smaller.

Positive selection modules
These modules are still in their initial design. 3x3 mm^2 Peltier elements from TE Technology (Traverse City, MI) were used. These elements exhibit linear heating temperature to applied voltage characteristics over a wide voltage range. The use of thermal paste between contact surfaces greatly enhances heating efficiency.

Accuracy of negative selection systems depends on binding efficiency; for 90% accuracy in a single reactor, theoretical studies suggested that the flow rate must be set so that it takes ~3h for solution to pass through it, assuming realistic concentrations and kinetic parameters. In contrast, accuracy is theoretically 100% under positive selection, although yield may be lower. All systems discussed below utilize positive selection. AND sorters must be implemented with reactors in series. OR sorters may be implemented with reactors in series or in parallel, but bias is lower in series. This permits creation of linear reactor arrays to solve complex expressions. Software to design instructions for operating arrays has O(P^2) complexity, where P is the number of pairs of parentheses in the expression; instructions can be implemented in a device in O(NL), or polynomial, time, where N is the

number of AND symbols in the expression, and L is the physical length of the system, which depends on the number of variables in the expression.

Conclusions: With new technologies emerging in the microfluidics field, more sophisticated systems can be designed and manufactured allowing reusable and reprogrammable DNA computers. Negative selection is possible, with variable parameters such as the reactor and bead size (the size of the beads determines the surface increase) and starting concentration, while positive selection has a lower error rate.

Microfluidic DNA-based computers theoretically have higher accuracy under positive selection than negative selection. AND and OR functions and entire Boolean expressions can be implemented by arranging reactors strictly in series. The operation of such a system is O(NL), so it can theoretically solve an NP-complete problem (satisfiability) in polynomial time.

Recommendations: By using a different architecture of the OR selector, it may be possible to skip the production of a prefabricated DNA-library and only manipulate the bit representations, allowing computations with more bits to be feasible. This would greatly enhance DNA-computing.

More refined simulations capable of predicting the behaviour of multi-reactor systems will be needed to help to test their efficacy.

Princeton University, Sohn Lab:
Introduction: The Sohn lab has been concentrating efforts in the "readout" aspects of this work. Readout here requires that we can detect single molecules of DNA and also the differently-sized nanocrystals (signifying the 0 and 1 bits) that tag the DNA.

Methods and Results:
Single-Molecule Sensing
We have developed an on-chip artificial pore device that is capable of electronically detecting single molecules of DNA and sizing sub-micron diameter colloids with nanometer precision (Saleh & Sohn, 2001, 2002, 2003a,b). Our on-chip pore can be fabricated with great ease and control using micromolding techniques and provides opportunities for diverse single-molecule detection applications beyond what is proposed for this particular grant.

Figure 2.1 shows one of our devices: a pore of length 3 μm and diameter 200 nm connecting two 5-μm deep reservoirs. Well-established lithographic and micromolding techniques are used to embed the pore and reservoirs into a polydimethylsiloxane (PDMS) slab. The slab is sealed to a glass substrate, and the device is wet with the solution to be studied. Molecules in the solution are electrophoretically drawn through the pore and partially block the flow of ions, leading to transient increases in the pore's electrical resistance. Molecular

sensing is accomplished with a four-point measurement of the electrical current through the pore using platinum electrodes patterned on the glass substrate.

To demonstrate the sensing capabilities of our nanopore, we have measured solutions of 2.5 μg/mL lambda-phage DNA in a 0.1 M KCl, 2 mM Tris (pH 8.4) buffer. Typical traces of measured current are shown in Figure 2.2. The striking downward peaks, ranging in size 10-30 pA and width 2-10 ms, correspond to individual molecules of DNA passing through the pore. In contrast, such peaks are absent when measuring only buffer. We further note that peaks are present only when using pores with diameters of 300 nm or less.

Previous work on colloids (Saleh & Sohn, 2001) has shown that, for particles of diameter much smaller than that of the pore, the ratio of the peak height to baseline current is approximately equal to the volume ratio of particle to pore: $\delta I/I = V_{particle}/V_{pore}$. We can estimate the volume of a single lambda DNA molecule by approximating it as a cylinder with a 2-nm radius (which include a 1-nm ionic, or Debye, layer) and a height equal to the contour length of the molecule (~16 μm). Given the known pore volume and a total current of $I =$ 15 nA, we can expect a decrease in current $\delta I \sim 30$ pA when a DNA molecule fully inhabits the pore. This estimate agrees well with the upper range of measured peak heights. Further corroboration for this model comes from the fact that no peaks are observed when using larger pores (pores > 300 nm in diameter). When a molecule inhabits a pore with a diameter > 300 nm, the expected response in current is less than 40% of that for a 200 nm diameter pore. Therefore, at 15 nA of current, the maximum peak heights for a lambda molecule will be less than 12 pA, a value not well resolvable above the noise. Our results thus suggest that the variation in peak size most likely corresponds to the different conformations of each molecule as it passes through the pore: maximum peak heights arise when an entire molecule inhabits the pores, whereas smaller peak heights occur when only a portion of a molecule resides within the pore. Future experiments will focus on controlling the conformation of each molecule in order to relate the measured peak size to the size of each DNA molecule. Such a relation has led to the precise sizing of nanometer-scale colloids; thus, our lateral nanopore device may be a viable alternative to standard gel electrophoresis for the coarse sizing of large DNA molecules.

Particle Sizing
Beyond sensing single molecules of DNA, we have another goal: to develop a sensor within a microfluidic channel that can size nanometer-scaled gold or semiconductor crystals the Alivisatos group (Berkeley) can fabricate and tag DNA. Using our on-chip artificial pore, we are capable of sizing *sub-micron* colloids with great accuracy and nanometer precision (Saleh & Sohn, 2001, 2002, 2003a,b). While sub-micron colloids are obviously larger than the nanocrystals the Alivisatos group can provide, our measuring their size with our pore represents a first step towards accomplishing this particular goal. In addition, as an "off-shoot" of this goal, we have developed an entirely new,

all-electronic technique for detecting the binding of *unlabeled* antibody-antigen pairs using our on-chip artificial pore (Saleh & Sohn, 2003b).

A particle passing through our on-chip artificial pore displaces conducting fluid causes a transient increase, or pulse, in the pore's electrical resistance that in turn is measured as a decrease in current. Because the magnitude of the pulse is directly related to the diameter of the particle that produced it (Saleh & Sohn, 2001, 2002), we can detect the increase in diameter of a latex colloid upon binding to an unlabeled specific antibody (see Figure 2.3). Using micron-sized pores embedded in polydimethylsiloxane (PDMS), we have successfully employed this novel technique to perform two important types of immunoassays: an inhibition assay, in which we detect the presence of an antigen by its ability to disrupt the binding of antibody to the colloid; and a sandwich assay, in which we successively detect the binding of each antibody in a two-site configuration (see Figure 2.4) (Saleh & Sohn, 2003b). The true strength of our technique is its *generality*: it does not rely on any functional properties of the free ligand. Thus, it can be applied to any ligand/receptor pair, provided the free ligand is large enough to produce a discernible change in the size of the colloid.

Conclusions: Overall, we have demonstrated that our on-chip artificial pore is a strong candidate for an integrated microfluidic device that can perform molecular computation. Three advantages are conferred upon our device. First, because our device is chip-based, only sub-microliter quantities of sample are needed and can be interrogated within minutes. Second, we utilize common microfabrication and micro-molding techniques to make the pore, reservoirs, and electrodes. This allows for quick and inexpensive device construction. Third, using chip-based fabrication can extend the device's capabilities by permitting either future integration of our measurement with other microfluidic components such as separation units or mixers, or construction of arrays of sensors on a single chip for performing many measurements or assays in parallel.

Berkeley, Alivisatos Laboratory:
Introduction: Our goal in this project has been to develop novel nanocrystal probes that enable detection of DNA in on-chip electrical assays. Our efforts have focused in two areas:

  i.   Methods to produce nanocrystals bearing precise numbers of oligonucleotides
  ii. Development of nanocrystal precise groupings that bear a collective signature

Methods and Results: In the area of nanocrystals bearing precise numbers of oligonucleotides, we have made steady progress. We have developed electrophoretic methods that allow us to separate nanocrystals bearing from

one up to eight oligonucleotides, and we have studied the range of conditions under which such separations are possible (for instance the dependence on nanocrystal size, oligonucleotide length, etc.

Conclusions: We find a rule of thumb as follows: separations of discrete conjugates are possible when the number of base pairs is ten times the diameter of the nanocrystal in length, e.g. 100 bp and 10 nm or less.

In the area of nanocrystal groupings we have successfully created a wide range of nanocrystal groupings, including nanocrystal dimers, trimers, and tetramers. We developed a general methodology for identifying these groupings and their yield based upon image analysis of transmission electron micrographs. We can analyze some 100,000 nanocrystal groupings and examine the pair distribution functions, allowing us to identify all species present. This tool is proving invaluable in boosting yields. We have recently extended this work to include mixed groupings of colloidal semiconductor quantum dots with metallic nanocrystals (these are the latest unpublished results). We have succeeded in preparing groupings comprised of a colloidal quantum dot with one, two, three, and four metal nanocrystals attached. It is expected that the Au nanocrystals will strongly modify the light emission from the colloidal quantum dot, and at some characteristic distances, the light emission will be greatly amplified by locally enhanced fields.
Conclusions: This new system could prove to be the single brightest light emitting probe, with excitation efficiencies orders of magnitude larger than any conventional dye or even dye-impregnated polymer bead.
This could have wide uses in military analytical biotechnologies, where background signals can overwhelm the emission form a few flurophores.

Stanford Laboratory:
Introduction: Rapid readout of DNA- or RNA-based computation would greatly decrease the time required to perform such computations. In addition to the electronic detection of the labelled molecules, the electronic data must be collected and analyzed.

Most current approaches for readout of the nucleotide-based computation requires the amplification of the DNA of interest using polymerase chain reaction (PCR). While PCR is a powerful tool that enables the sensitive detection of non-abundant DNA molecules in a sample, several drawbacks exist. False-positive signals can result from a non-specific or otherwise inaccurate hybridization event. Although this can be a rare occurrence, PCR amplification causes the event to be detected. False-negative signals also can result from inefficient polymerization during PCR due to factors such as some sequences being more difficult to amplify than others. One way to avoid the need for PCR amplification and lower the occurrence of false-positive and false-negative events is to develop a tool that can detect single molecules of DNA.

Several groups are developing single-molecule detection methods using a nanopore (Deamer and Akeson, 2000; Deamer and Branton, 2002; Li et al., 2003). The first efforts in this field used an ion-channel protein to form the nanopore, and the ionic current through the pore was measured. The first reported success was the ability to detect single oligonucleotides moving through the pore due to the blockage of ionic current while the oligonucleotide travelled through the nanopore (Kasianowicz et al., 1996). This overcomes the common molecular diagnostics drawback of requiring multiple copies of the analyte, since single molecules were detectable. The ultimate goal of these efforts is to discriminate individual nucleotides in a DNA molecule based on differential blockage of the ionic current. However, to date no approach has overcome the limits on resolution to detect the small differences in electronic signals between the nucleotides. Part of the barrier includes the analysis of the signals resulting from the detection. As part of this effort, Stanford has explored data analysis to contribute towards the electronic identification of single molecules for readout of nucleotide-based computation.

Methods: Stanford's effort requires the application of software design principles combined with the mathematical analysis of acquired data. Stanford has focused on basic automated signal analysis, with the goal of having a software module that will take raw signal data and analyze and summarize events.

Described below are the components for the acquisition and analysis modules needed to accelerate Stanford research efforts in that of single-molecule detection. The software has been written in Microsoft Visual Studio with Visual Basic and Visual C/C++ languages. The National Instruments (NI) Measurements Studio 7.0 controls and library have also been used. Implementation of the basic event detection algorithms has been described previously (Colquhoun and Sigworth, 1995, pp. 490). These standard algorithms are based on amplitude threshold detection and time course fitting.

Results: As part of this effort, Stanford has:
- Developed software modules for configuration of initial acquisition and pulse parameters for National Instruments acquisition boards
- Developed export modules of signals to spreadsheet and XML format for further manipulation and analysis
- Developed synchronization protocol for National Instruments acquisition board to generate voltage pulse and triggers for external devices
- Automated and optimized the saving procedure of acquired signals
- Designed new chamber setups for nanopore measurements with and without the help of patch pipettes to achieve significantly lower noise in current signal

Conclusion: The preliminary software modules for the automated acquisition of data have been developed and are ready for the acquisition of electronic

signals. During the implementation of the National Instruments library software modules in a single application, it became clear that an object oriented approach for such implementation has limitations. The recommended solution is described below. The software modules have been tested on data generated from a separate Stanford effort. Additional data are required for the full use of the developed software and continued development to add additional features to the software.

Recommendations: Due to the limitations as mentioned above, the full use of the capabilities of a new 10MHz Gage acquisition board requires the creation and integration of new modules is required. The graphical interface and database access modules should be the same. The new Gage acquisition board will have to be synchronized with the National Instruments board, the only board able to generate pulse and trigger signals and perform general long time acquisition of nanopore signals. The faster Gage acquisition board has to be triggered and synchronized with other devices in the case of a specific event recognized by National Instrument board or by the Gage board itself. The implementation and evaluation of new design of chamber setup for nanopore measurements needs to be continued and critically examined to reduce the noise.

The setup for the electronic detection of samples is the critical component of this effort. As more data become available, additional modifications of the software will be done.

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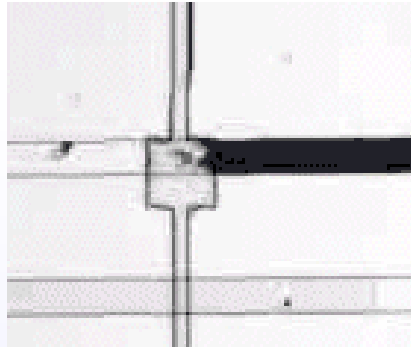
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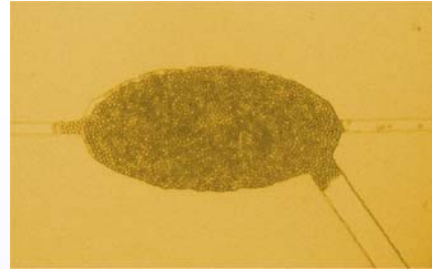
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Figure 1.1.   *A microscope image of* (a) *a valve. 5.6 μm beads were collected before a partially closed valve. The flow channel is 100 μm and the valve pad is 200x200 μm. (from van Noort* et al., *2003)*

*(b) an ellipsoidal microreactor within a 200x450 μm rectangle filled with 5.6 μm beads. From left to right is a 25 μm flow channel, while in entering from the bottom, right hand corner is the 50 μm wide bead delivery channel.*
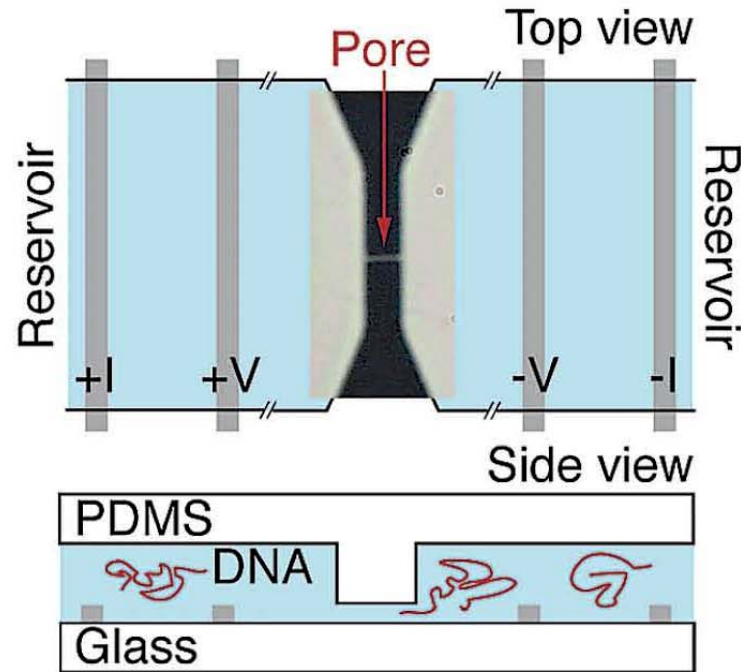
Figure 1: Schematic top and side views of our on-chip artificial pore device, which consists of two 5 μm-deep reservoirs connected by a lateral pore of 3 μm length and 200 nm diameter; an optical image of an actual pore sealed to a glass coverslip is incorporated into the top view. Molecules in the reservoirs are electrophoretically drawn through the pore, partially blocking the flow of ions. The current through the pore is measured using a four-terminal technique, where the voltage and current controlling the platinum electrodes are as labeled.
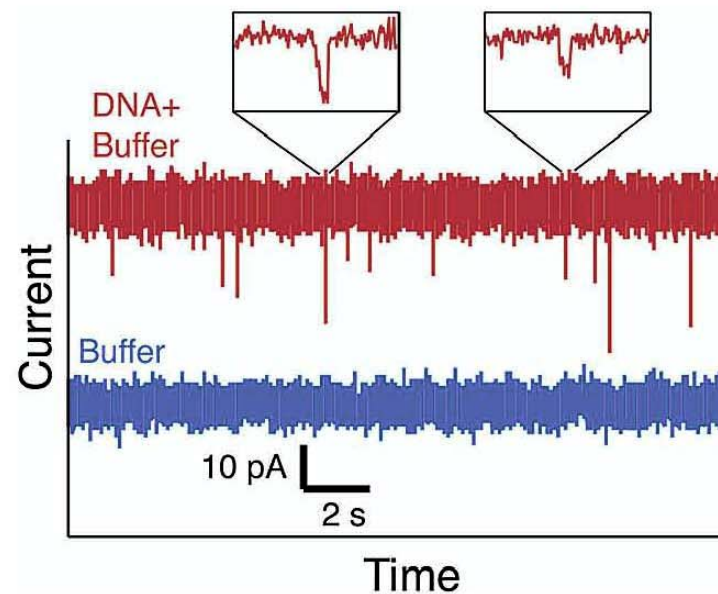
Figure 2: Typical traces of current vs time for solutions of buffer (lower trace) and buffer with lambda phage DNA molecules (upper trace) when 0.4 V is applied across the pore. The traces are offset for clarity; the total current in each case is ~ 15 nA. Each downward spike in the lower trace represents a DNA molecule passing through the pore. The spikes are typically 2-10 ms in duration and are well resolved, as shown in the insets. The variations in peak height most likely correspond to the different conformation of each molecule.
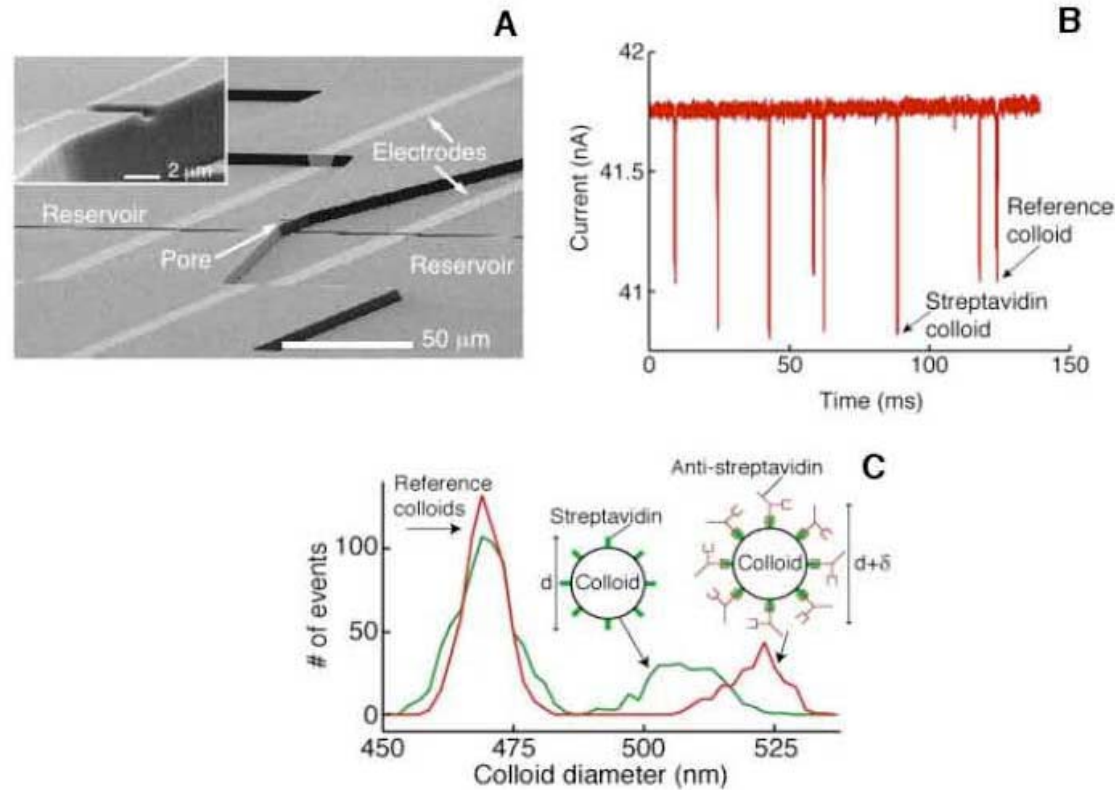
Figure 3: **(a)** SEM photo of an artificial micron-sized pore in quartz. **(b)** A typical measurement of the current across a pore as different colloids pass through it. Each downward pulse corresponds to a single colloid transiting the pore. **(c)** A histogram showing the distribution of colloid diameters measured from a solution that contains only reference and streptavidin colloids (green line), and a solution that contains both types of colloids and 0.1 mg/mL of monoclonal anti-streptavidin antibody (red line). The specific binding of anti-streptavidin to the streptavidin colloids produces a clear increase in the diameter of the colloids.
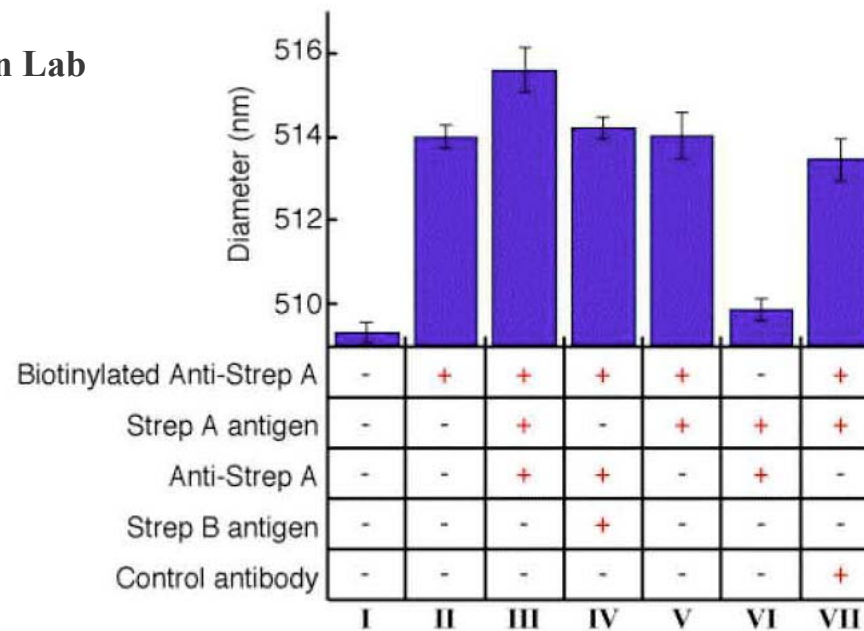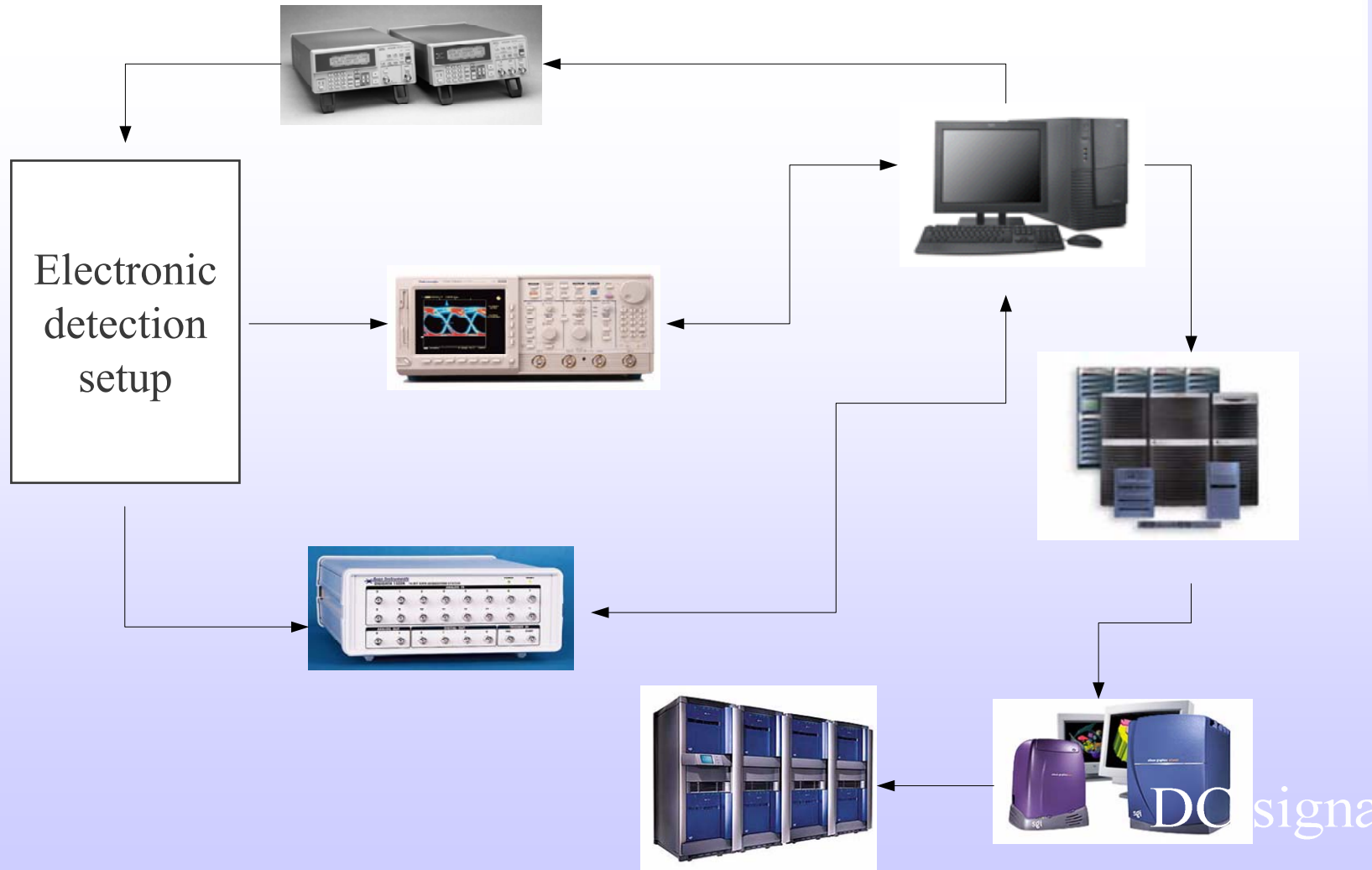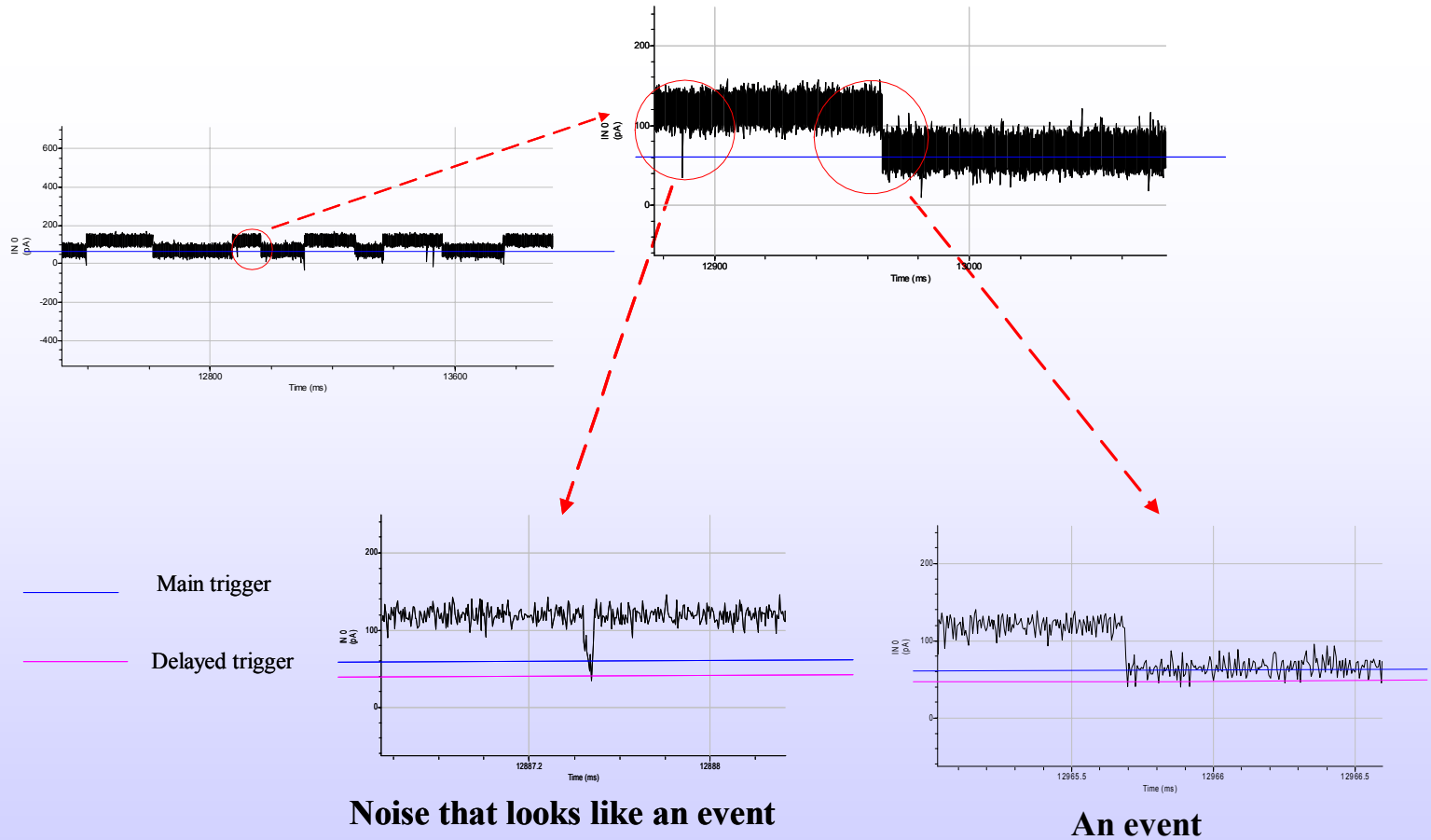
Figure 4: Summary of the mean colloid diameters measured when forming an antibody-antigen-antibody 'sandwich' on the colloid surface. All solutions contain the reference and streptavidin colloids in a 0.5x PBS buffer (pH 7.3), along with additional components as indicated by the '+' in the column below the plotted bar. Column I indicates the measured diameter of the 'bare' streptavidin colloid. We measure a ~ 5 nm increase (column II) in diameter after conjugating a biotinylated antibody (biotinylated anti-Streptococcus Group A) to the streptavidin coated colloids. A further increase of ~1.6 nm is seen (column III) when adding both extract from a culture of Streptococcus Group A and a secondary antibody specific to that antigen (unlabeled anti-Streptococcus Group A); this increase indicates the formation of the sandwich on the colloid surface. The specificity of the configuration is shown by the lack of an increase in diameter when adding extract from a culture of Streptococcus Group B (which is not bound by either antibody) in place of the Group A extract (column IV), or an irrelevant antibody in place of the specific secondary antibody (column VII). When adding the specific antigen and secondary antibody to unconjugated colloids (column VI), we measure no significant diameter increase, indicating that non-specific adhesion of antigen-secondary antibody complexes are not the cause of the diameter increase seen in column III. Finally, when adding the specific antigen alone to the conjugated colloids (column V), we see no increase in diameter, indicating that the diameter increase in column III is primarily due to the binding of the secondary antibody.
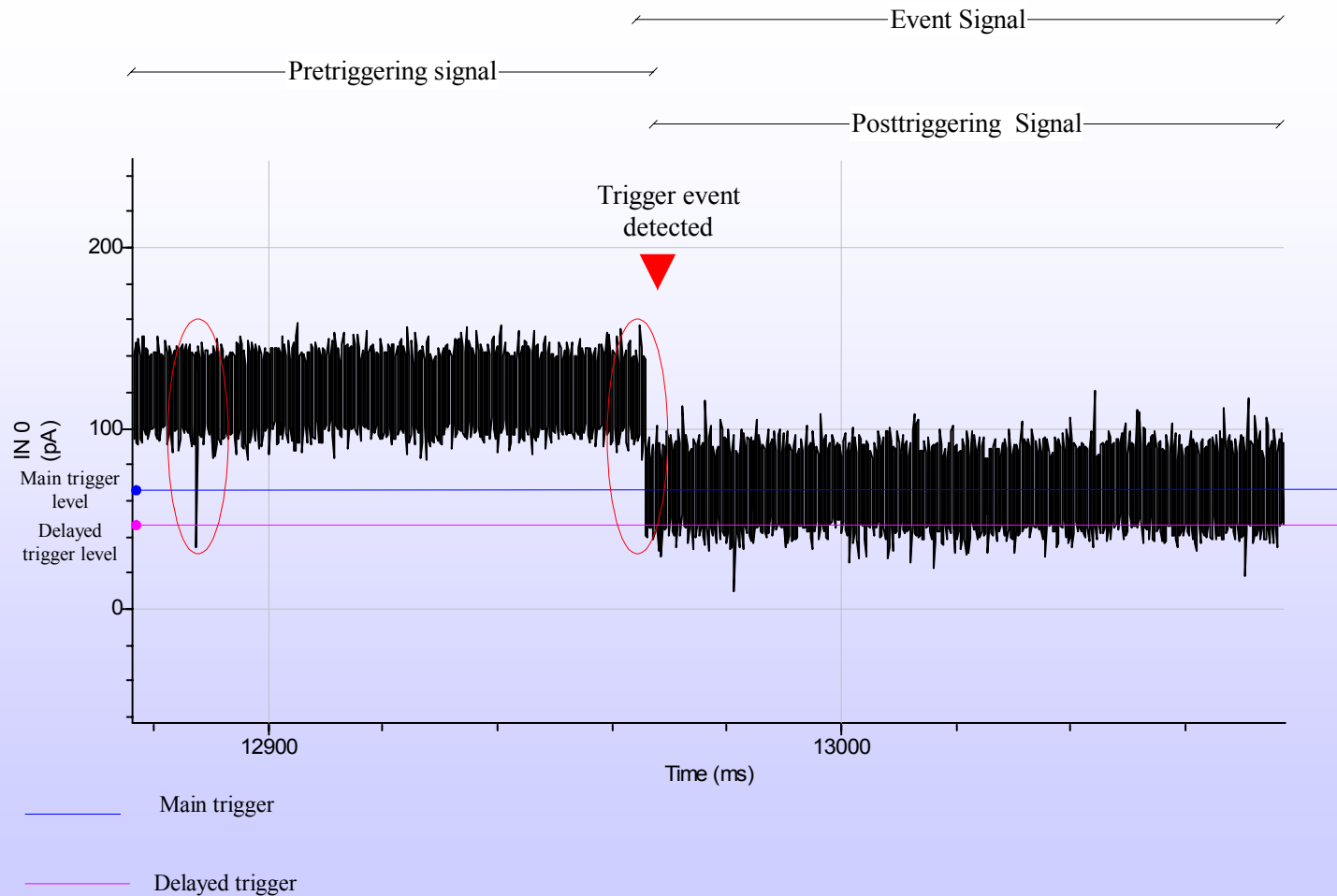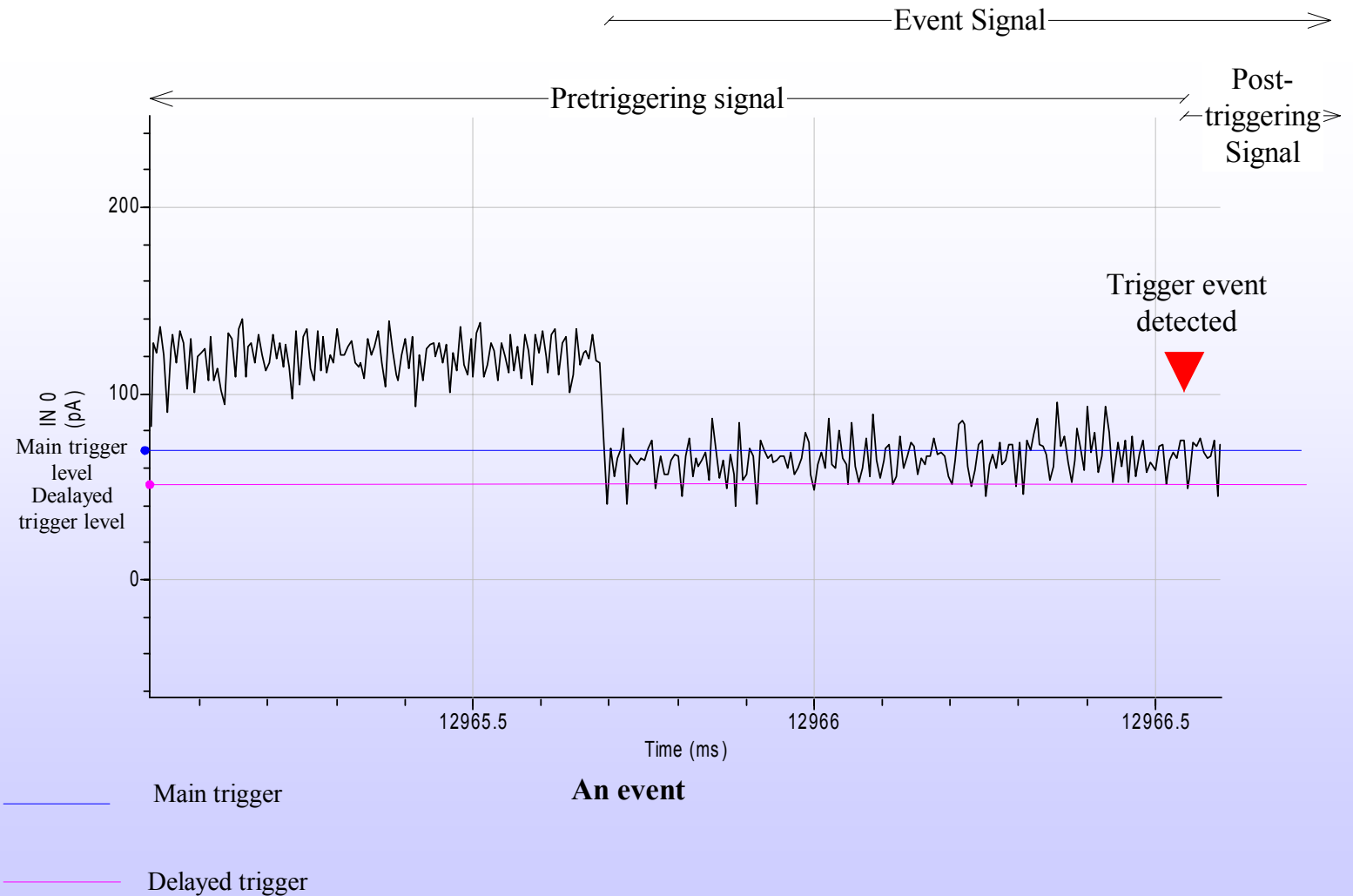
System

Triggering System

Main trigger

Delayed trigger

Noise that looks like an event

An event

Example of Event Signal

Trigger detected

Event Signal

Pretriggering signal

Post-triggering Signal

200

Trigger event detected

IN 0 (pA)

100

Main trigger level
Dealayed trigger level

0

12965.5   12966   12966.5

Time (ms)

_____   Main trigger   **An event**

_____   Delayed trigger

Noise Rejection

Noise that looks like an event

_____   Main trigger

_____   Delayed trigger